PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Dan Veniamin NICOLAU, et al.

Appln. No.: 10/689,738

Confirmation No.: 4321

Filed: October 22, 2003

Docket No: Q78126

Group Art Unit: 2878

Examiner: Unknown

For: MICRO/NANO-STRUCTURES FABRICATED BY LASER ABLATION FOR MICRO-ARRAY APPLICATIONS

SUBMISSION OF PRIORITY DOCUMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith is one (1) certified copy of the priority document on which a claim to priority was made under 35 U.S.C. § 119. The Examiner is respectfully requested to acknowledge receipt of said priority document.

Respectfully submitted,

Bruce E. Kramer
Registration No. 33,725

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Enclosures: Australia 2002952384

Date: April 22, 2004

Patent Office
Canberra

I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002952384 for a patent by SWINBURNE UNIVERSITY OF TECHNOLOGY as filed on 31 October 2002.

WITNESS my hand this
Thirtieth day of October 2003

J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

Dan Veniamin NICOLAU, et al.
USSN: 10/689,738
Bruce E. Kramer 202-663-7951
1 of 1

THIS PAGE BLANK (USPTO)

Structures

Field of the Invention

The present invention relates to structures suitable for use in arrays, and in particular but not exclusively, to structures having combinatorial surfaces that allow molecules to attach to a localised area of the surface according to the characteristics of the localised area. The invention also relates to methods of fabricating structures having combinatorial surfaces and to their use in arrays and assays.

Background of the Invention

Micro-structures are fabricated and used in DNA microarrays and microassays which may provide a rapid and moderate cost biosensing system, for example, for detecting DNA base-pairing or hybridisation. Microarrays are orderly arrangements of samples deposited on a micro-structure. The typical size of a sample spot in a microarray is less than 200 microns and each microarray may hold thousands of samples.

Technologies available for the fabrication of these structures must ensure the confinement of different sample molecules in localised areas, which may be flat or profiled. Technologies that are available for fabricating these structures include (i) spotted-array-based methods, De Wildt *et al.* (2000), Walter *et al.* (2000); (ii) soft lithography, Zhao *et al.* (1997), Bernard *et al.* (1998) (iii) photolithography, Fodor *et al.* (1991), US 5391463, Nicolau *et al.* (1998), Nicolau *et al.* (1999); (iv) scanning probe lithography, Wadu-Mesthrige *et al.* (1999); (v) laser or ion-beam ablation, Schwarz *et al.* (1998), US 5858801, and (vi) microfabrication of profiled features for e.g. microfluidic devices, Wang *et al.* (2000), Sundberg (2000), Nicolau and Cross (2000), McDonald *et al.* (2001), Ismagilov *et al.* (2001). These methods have been listed, not comprehensively, in the order of their '3D-ness', that is, starting with features that are elevated above the surface by a few nanometers (methods i, ii, and iii); to quasi-flat features (methods iv and v); and ending with samples that are placed on the bottom of etched or developed micro-features (methods v and vi). While some types of biodevices dictate a particular design of the biodevices (eg. microfluidics devices require profiled channels) others do not (eg.

microarrays normally have a flat surface). The profiled features of methods (v) and (vi) have the advantage of minimization of inter-spot contamination and the drawback of difficult access of the recognition component (eg. antigen for antibody microarray) in a micro-defined area.

Two potentially important surface-related problems of this technology are (i) the possible difference between the surface concentration of different molecules on the same surface and (ii) the possible surface-induced denaturation of the structure and subsequently the change of the bioactivity of the adsorbed biomolecules, Andrade and Hlady (1991).

Economic requirements dictate the preference for use of a minimum amount of material for fabrication and operation of the micro-assay. While classical microarray technology involves flat surfaces, with inherent spread of the small volume of the analyte solution in the deposited droplet, a profiled microfabricated location in which the droplet is deposited would be a more efficient solution. However, the depth of the profiled feature has to be minimized in order to allow free diffusion of the recognition molecule in the micro-fabricated well.

Among the many procedures for microfabrication, ablation has the advantage of a step-wise process without the involvement of fluids such as in microlithography. In principle, there are few possibilities to fabricate the micro-wells, each of which have advantages and drawbacks. A first possibility is the ablation of a protein-blocked single layer of a polymer, Schwarz *et al.* (1998), US 5858801, which is preferably designed to promote molecule adsorption, especially proteins, without surface-induced denaturation. This is the simplest choice. However, this approach requires either expensive laser ablation tools operating in deep-UV (e.g. 248nm) and non-fluorescent polymers (e.g. PMMA), or the use of more convenient (e.g. near-UV) lasers and polymers that absorb in that region, but which are likely to interfere with the detection through background fluorescence. A second possibility is adopting a bilayer structure with an ablatable layer on the bottom and a molecule-adsorbing, sacrificial layer on top. However, experiments proved that the ablated material (e.g. Au) can not be efficiently released during the ablation

through the top polymeric layer, which leads to the frequent peel-off of large areas of the bilayer structure. A third possibility is to deposit a very thin ablatable layer on top of a molecule-adsorbing, transparent to laser wavelength, non-ablatable polymeric layer. This technological avenue raises the issue of the fate of the physics and chemistry of the top surface of the bottom layer, which is exposed to large amounts of energy during the ablation of the top layer. The logical approach would be to tune the ablation in a manner that will preserve the bottom layer. Unfortunately there is only a remote possibility that this can be achieved.

Among the enabling technologies for the above patterning methods, laser beams are capable, according to the exposure energy and the sensitivity or absorbance of the exposed material, to enable both photolithography and photo-assisted etching. Also, focused laser beams can, in principle, solve a critical fabrication and operating problem of the structures better than most other alternative methods, i.e. they may provide controlled and confined variation of the surface properties of the areas upon which different molecules are adsorbed.

Advantageously, one embodiment of the present invention may provide the fabrication via laser ablation of shallow-profiled structures with surfaces having areas tailored to accommodate an universal adsorption of molecules.

A further problem associated with the use of arrays is the identification of different samples within the array, or the identification of different test samples that are applied to the array in a assay. Advantageously, at least one embodiment of the present invention provides an 'informationally-addressable' structure or an array where information about each sample in the array or each test sample applied to the array in a assay is encoded by the combination of shallow-profiled features within the array.

Summary of the Invention

According to one aspect of the present invention there is provided a structure comprising (i) a first layer comprising a molecule-adsorbing, substantially non-ablatable material, and (ii) a second layer comprising an ablatable material, wherein the second layer is disposed on the first layer and wherein at least a portion of the second layer has been ablated to expose a surface of first layer.

Preferably the exposed surface of the first layer comprises at least two localized areas having molecule-adsorbing capacities for molecules with different adsorbing properties. In another preferred embodiment, a plurality of portions are ablated to form an informationally-addressable pattern.

According to another aspect of the invention there is provided an array comprising (a) a micro-structure which comprises (i) a first layer comprising a molecule-adsorbing, substantially non-ablatable material, and (ii) a second layer of ablatable material, wherein the second layer is disposed on the first layer and a plurality of portions of the second layer have been ablated to expose a surface of the first layer and thereby form a plurality of profiled features, and (b) at least one molecule adsorbed on the surface of the first layer in at least one of the plurality of profiled features.

In a further aspect, the present invention provides a method of fabricating a structure as described above, comprising the steps of:

(a) obtaining a substrate supporting (i) a first layer comprising a molecule-adsorbing, substantially non-ablatable material and (ii) a second layer comprising a non-ablatable material disposed on the first layer;

(b) laser ablating at least a portion of the second layer to expose a surface of the first layer.

In yet a further aspect of the invention there is provided an assay method comprising the steps of:

(i) contacting an array described above with a test sample that may contain an analyte that binds to the at least one molecule adsorbed on the surface within the at least one profiled feature;

(ii) detecting the binding of the analyte and the adsorbed molecule.

Detailed Description of the Invention

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

The term "molecule-adsorbing" as used herein refers to materials and surfaces capable of binding molecules. The molecules may be bound to the surface or material by any interaction which is capable of maintaining the molecule in contact with the surface or material. For example, the surfaces or materials may bind molecules by ionic interactions, electrostatic forces, hydrogen-bonding or hydrophobic interactions. Alternatively, the surfaces or materials may bind molecules by the formation of covalent bonds.

The structures of the present invention are preferably micro-structures for use in microarrays and micro-assays. As used herein the term "micro" means small. A micro-structure may range in width from a few microns to millimeters and may contain thousands of profiled features, each profiled feature having a width or diameter in the range of submicrons to 10s of μms.

The first layer comprising a substantially non-ablatable material may be any material capable of adsorbing molecules. Preferably the first layer is substantially

transparent to laser wavelength. Preferably the first layer is polymeric and preferably the polymer is capable of thermal degradation under laser ablation conditions to provide a surface having diverse surface properties. Especially preferred are polymers that thermally degrade to provide a surface having localized areas which are hydrophobic, hydrophilic, acidic, basic, charged or neutral. Suitable polymeric materials include polyacrylates, polycarbonates, polystyrenes, fluorine-containing polymers, polyethylenes and their derivatives. Examples of suitable polymers include polymethylmethacrylate (PMMA), polyacrylic acid, polyacrylonitrile, polymethacrylate, styrene-acrylonitrile co-polymers, butadiene-styrene copolymers, polyalkylstyrenes fir example polymethylstyrene, polyethylstyrene and polypropylstyrene, and polytetrafluoroethylene (PTFE). Particularly preferred is PMMA.

In a preferred embodiment, the surface of the first layer that is exposed has at least two and preferably a plurality, of localized areas having molecule-adsorbing capacities for molecules with different adsorbing properties. For example, each localized area may present a hydrophobic, hydrophilic, acidic, basic, charged or neutral surface and therefore has the capacity to adsorb molecules which have a surface that is complementary or attracted to the localized area of the surface. A localized area of hydrophobicity will adsorb molecules that also have a hydrophobic surface, whereas a localized area which has a negatively charged surface will adsorb molecules having a positively charged surface. The term "molecule-adsorbing capacity" refers to the surface properties presented by a localized area.

The localized areas may be formed in a structured or unstructured manner. For example, a structured surface where the localized areas form a predetermined pattern on the surface first layer may be formed. Such a pattern may be, for example, alternating localized areas of hydrophobic and hydrophilic surfaces and surfaces with different chemistries (eg: NH_2, CO_2H, OH).

Alternatively, the surface may be unstructured where the laser ablation method used results in areas having different molecule-adsorbing capacities. For example, areas of

hydrophobicity and hydrophilicity may be obtained depending on the amount of thermal energy to which a particular area is exposed. For example, and without being bound by theory, based on AFM topography and lateral force imaging, as well as the knowledge regarding laser ablation, the following mechanism of formation of the observed structures and subsequent variations in molecule adsorption may be proposed. Laser exposure (ns) causes the overheating of a polymer to a point where the polymer is melted and chemical reactions start to occur. The expected reactions would be, in the order of increasing pyrolysis temperature, (i) the termination of the side ester groups at one of the C-O bonds, resulting in a more hydrophilic material; (ii) depolymerization of the main chain, preserving the same hydrophobicity; and if the process is quick enough (iii) the breaking of the side bonds, resulting in a more hydrophobic material. Therefore, we can hypothesize that there are three regions in the micro-well. At the center of the ablated line where the thermal energy would reach a maximum the decomposition is the most advanced, the polymer would experience the breaking side chain C-C groups, resulting in a more hydrophobic material. Between the center and the edge of the ablated line the polymer undergoes depolymerization only. At the edges of the ablated line, where the thermal energy has the lowest levels and the remaining metal layer absorbs the overheating, the polymer is de-esterified (with generation of gases), melted and expulsed over the edges of the micro-well, resulting in a porous, more hydrophilic zone. Therefore an unstructured surface in a profiled feature may have a central hydrophobic area and a hydrophilic area at its edges.

The second layer comprising an ablatable material may be any material that is opaque to laser wavelength and is able to be evaporated under ablation conditions. Preferably the ablatable material is a metal that can be deposited in a thin layer. Suitable metals include Au, Cr, Ag, Mg, Ti, V, Mn, Fe, Co, Ni, Cu, Zn, Cd, Pt, Pd, Rh, Ru, Mo, W and Pb. Particularly suitable ablatable materials include Ag, Cr, and Au. Particularly preferred is Au.

Laser ablation of at least a portion of the second layer exposes the top surface of the first layer and may cause thermal degradation of the localized areas of the exposed

surface of the first layer. The laser ablation of the second layer forms shallow profiled features in the structure. The shallow profiled features may be any shape but are preferably square or rectangular and may be in the form of a well or a channel. The bottom of the profiled feature is formed by the top surface of the first layer. The depth of the shallow profiled feature corresponds to the thickness of the ablatable layer and is preferably less than 100nm, more preferably 50nm or less.

Preferably the structure comprises a further blocking layer. The blocking layer may be any material capable of preventing binding of molecules. The blocking layer is ablated together with the second layer. However, the blocking layer remaining on top of the second layer after ablation repels the molecule to be adsorbed on the surface of the first layer or the analyte or recognition molecule from the non-ablated portions of the second layer. The blocking layer may include any protein that is unreactive and will not interact with the molecule to be adsorbed or their complementary components. Suitable blocking materials include polyethylene glycol, polyethylene oxide and inert proteins such as bovine serum albumin (BSA).

In a preferred embodiment, the structure includes an orderly arrangement of a plurality of profiled features. The plurality of profiled features may form a plurality of wells or a plurality of channels. In a particularly preferred embodiment, the plurality of profiled features may be arranged in a pattern that is capable of identifying a feature of an array formed from the structure. For example, a plurality of channels may be formed in a "bar code" type arrangement and each structure may contain a plurality of different bar code type arrangements. Each bar code type arrangement may be used to encode particular information about an array prepared from the structure or the samples applied to the array in an assay. The term "informationally-addressable" as used herein refers to the ability of the profiled features to encode information about an array or an assay.

In one embodiment, each informationally addressable profiled feature or bar code may be used to identify a different molecule adsorbed on the surface of the first layer in a profiled feature of an array or may be used to identify a series of different concentrations

of a single molecule adsorbed on a respective series of bar code type arrangements. Alternatively, each bar code arrangement may be used to encode information about an assay in which the array is to be used. For example, the bar code may be used to identify the source of the analyte or recognition component. In a diagnostic assay where each profiled feature forms a bar code and each profiled feature has the same molecule, eg. a protein or gene, adsorbed on the exposed surface of the first layer, the bar code could be used to identify the patient who is being tested.

The structure of the invention preferably further comprises a substrate that supports the bilayer comprising the first and second layers. The substrate can be made of any material suitable for supporting the first and second layers and which is capable of withstanding the conditions used in preparing and using the structure. Examples of suitable substrates include quartz glass, mesoporous silica, nanoporous alumna, ceramic plates, glass, graphite and mica. Preferably the substrate is ordinary glass. Alternatively, the substrate may be part of the apparatus used for fabricating the structure or array, or for performing the assay. The structure may be prepared on the surface of a substrate and then removed or transferred to another substrate.

The structure of the invention may be used in the preparation of an array, where at least one profiled feature has a molecule adsorbed on exposed surface of the first layer. Preferably the structure has a plurality of profiled features and each profiled feature contains a molecule adsorbed on the exposed surface of the first layer. Each profiled feature may contain the same or a different molecule.

The molecule adsorbed on the molecule-adsorbing surface of the profiled feature may be any molecule of interest. For example, the molecule may be a biomolecule such as a gene, oligonucleotide, protein, peptide or polysaccharide. Alternatively, the molecule could be a drug or potential drug derived from natural or synthetic sources.

The present invention is particularly useful for enabling the adsorption of proteins. Proteins present extremely varied molecular surfaces, for example, hydrophilic,

hydrophobic, acidic, basic, neutral or charged surfaces, and may be sensitive to denaturation upon adsorption on a surface. The variation in the molecule-adsorbing capacity in the surface of the first layer provides localized areas that may interact with different proteins having different surface properties or may interact with the same protein by a different surface. This latter interaction will ensure that at least some of the adsorbed protein will have a recognition site, such as an active site, receptor or binding site, exposed for use in an assay. The immobilized proteins may be used to probe protein-protein, enzyme-substrate, protein-DNA, protein-oligosaccharide or protein-drug interactions.

The arrays of the invention may be used in assays to probe interactions between an adsorbed molecule and an analyte or recognition component. Such interactions include DNA-DNA, DNA-protein, DNA-drug, DNA-oligosaccharide, protein-protein, enzyme-substrate, protein-DNA, protein-oligosaccharide, oligosaccharide-protein, oligosaccharide-oligosacchride, oligosacchride-drug or drug-drug interactions. For example, an assay may be used to find substrates or inhibitors of a particular enzyme adsorbed on the exposed surface of the first layer, or may be used to determine a mechanism, such as whether the enzyme inhibitor is competitive or non-competitive.

The assay method of the invention may be performed by contacting the adsorbed molecule(s) in an array with a test sample, the test sample potentially containing an analyte or recognition component that will bind to the adsorbed molecule. The presence or absence of the analyte or recognition component in the test sample can then be detected. The term "analyte" or "recognition component" as used herein refers to a molecule that is recognised by and interacts with the molecule adsorbed in the profiled feature.

The adsorbed molecule and analyte or recognition component may be selected from pairs of complementary compounds such as a single strand of DNA, RNA or an oligonucleotide and their complementary strand, an antibody and an antigen, an enzyme and a substrate or an inhibitor, a drug and a receptor.

The coupling of the adsorbed molecule and the complementary component may be detected by any detection means known in the art. For example, fluorescence detection may be used, where a fluorescent marker is tagged onto the adsorbed molecule or the analyte or recognition component or may be bound to the adsorbed molecule/analyte or recognition component pair in a further step. Preferably the marker is tagged onto the analyte or recognition component or may be bound to the adsorbed molecule/analyte or recognition component pair in a further step. Other suitable means of detection includes the use of luminescent, phosphorescent or radioactive markers or the use of nanoparticles or magnetic beads.

The assay may be used as a diagnostic assay or may be used in high throughout screening of molecules. For example, in a diagnostic assay the array containing many different DNA molecules indicative of specific genes may be prepared and a test sample from a patient, for example, serum, added to the array by "flooding" the array and then after appropriate washing and the addition of a detection marker, the coupling of complementary DNA sequences can be detected. This may give an indication of whether the patient has a specific gene or a mutation in a specific gene.

Alternatively, an array of profiled features all containing the same antigen or antigen, could be prepared. Test samples obtained from different patients suspected of having a particular disease caused by the antigen, could be added, one test sample to each of the profiled features. After washing and addition of an appropriate marker, the coupling of antigen and antibody can be detected. The samples in which an interaction between antigen and antibody are detected, can be used to indicate the presence of the disease state in the patients providing the samples.

The present invention may be adapted for use in known genetic and protein assays. Preferably the assays are protein assays such as antibody assays.

In another embodiment the assay may be used in high throughput screening. For example, an array containing a variety of drug targets which are known to be involved in

the initiation or progress of a disease state, can be prepared. The array can be contacted with a potential drug and its interaction with the drug targets assessed.

The structures and arrays may be prepared by obtaining a substrate supporting a first layer comprising a molecule-adsorbing, substantially non-ablatable material and a second layer comprising an ablatable material disposed on the first layer. The substrate may be coated with the first layer by any suitable technique, for example, sputter coating, spin coating. Preferably the first layer is coated on the substrate by spin coating.

Similarly the second layer may be applied to the first layer by any suitable means. For example, sputter coating, spin coating or electroplating. Preferably the second layer is applied by sputter coating.

Optionally the surface of the second layer is blocked by the application of a blocking layer. For example, the blocking layer may be an inert polymer, such as polyethylene oxide or polyethylene glycol, or an inert protein, such as BSA. A protein blocking layer may be applied by immersion and incubation of the substrate supporting first and second layers in a solution of blocking protein (1-5% w/v BSA in an appropriate buffer) at room temperature for 15 minutes to 1 hour or by soaking in the protein solution or via addition of a droplet of a protein solution. A polymeric blocking layer may be applied by spin or sputter coating.

The second layer is then subjected to laser ablation such that at least a portion, and preferably a plurality of portions, of the second layer is ablated to expose the surface of the first layer.

The fabrication of structures and arrays according to the invention are shown in Figure 1 and 2. In Figure 1, a substrate (1) coated with a first layer comprising a molecule adsorbing, substantially non-ablatable material (2), a second layer comprising an ablatable material (3) and a blocking layer (4), is subjected to laser ablation to produce a profiled

feature (5) to which a droplet of molecule (6) to be adsorbed is added with a picoliter pipette (7). In Figure 2, an array of different biomolecules is prepared.

The laser wavelength used for ablation may be between 100nm to 1200nm, preferably 150nm to 1100nm. A typical high energy wavelength is in the range of 150 to 300nm. A typical low energy wavelength is in the range of 300nm to 1100nm.

Preferably the fabrication platform consists of a computer controlled laser ablation system, comprising a research-grade inverted optical microscope, a pulsed nitrogen laser emitting at 337nm, a programmable XYZ stage and a Pico-litre pipette mounted on the XYZ stage. Preferably the profiled features formed during the laser ablation step are micro-wells or micro-channels.

Wells having diameters of from sub-micron widths to about 50μm are able to be prepared and are useful in preparing arrays. Wells having diameters of 5-20μm, 1-5μm and submicron widths are readily achieved by focussing through a 20 x dry objective, a 40x dry objective or a 100x oil immersion lens, respectively. Preferably the wells have widths in the range of 5μm-50μm, more preferably 5μm to 10μm.

Channels having from submicron widths to about 50μm widths may also be prepared. The channel may be any length but is preferably 5 to 200μm long. In a similar manner to wells, channels having diameters of 5-20μm, 1-5μm and submicron widths are readily achieved by focussing through a 20 x dry objective, a 40x dry objective or a 100x oil immersion lens, respectively. Preferably the channels have widths in the range of 5-50μm, more preferably 5-10μm.

In a preferred embodiment the XYZ stage is programmed to allow the laser ablation of vertical lines forming channels at different distances to form a pattern that is informationally addressable.

A structured surface may be prepared by exposing a number of localised areas to laser exposure with different exposure times, different powers, and/or different wavelengths, all translating in different energies absorbed by the ablatable material. Additionally, the thickness of the ablatable material may be varied, therefor requiring different energies of ablation. A first localised area may be exposed to a high energy wavelength for a short period of time, in the order of femtoseconds, resulting in ablation of the ablatable material but minimal build up of thermal energy and therefore minimal decomposition of the surface of the molecule-adsorbing, non-ablatable material. A second adjacent localised area may be exposed to a lower energy for a longer period of time, for example in the order of nanoseconds to microseconds, resulting in a greater buildup of thermal energy and therefore greater thermal decomposition of the surface of the molecule-adsorbing, non-ablatable material occurs. The process may be repeated to provide a number of localised areas having different or alternating adsorbing properties on one surface.

The array of the present invention may be prepared by adsorbing molecules of interest onto the exposed surface of the first layer within the profiled feature. One method of fabricating the array is to laser ablate a plurality of portions of second layer to form a plurality of profiled features and then to "flood" the structure with a solution containing the molecule to be adsorbed. This method provides an array having the same molecule adsorbed in each profiled feature. Another method of fabricating the array is to laser ablate a plurality of portions of the second layer to form a plurality of profiled features in a "spatially-addressable" mode and then deposit the molecule to be adsorbed in each of the profiled features with a Pico-liter pipette. This technique may be used to achieve an array having a different molecule adsorbed in each profiled feature or at least some profiled features having different molecules adsorbed in them. As used herein the term "spatially-addressable" refers to the ability to apply a solution to an individual profiled feature.

In a similar manner when performing the assay, the test solution of analyte or recognition component may be applied to an array containing different molecules in at

least some of the plurality of profiled features, by flooding the array with the test solution containing the analyte or recognition component. Alternatively, different test solutions containing different analytes or recognition components may be applied to each profiled feature using the spatially addressable mode described above.

Brief Description of the Figures

Figure 1 schematically represents the procedure for preparing structures and arrays by laser ablation.

Figure 2 schematically represents a procedure for fabrication of anablated array, with fluorescent images before (middle top and middle bottom) and after antibody deposition (right). The ablated micro-wells are 100 x 100 μm.

Figure 3 represents fluorescence images of anti-chicken IgG AlexaFluor 546-conjugate deposited in profiled features prepared by laser ablation of Au deposited on PMMA. The profiled areas were prepared using different laser doses. Upper left area ablated with 60% laser power, bottom left – 100%, upper right – 40% and bottom right- 80%.

Figure 4 represents topographical (left) and friction force (right) images of a Au-PMMA bilayer struction exposed to different laser doses. Upper left area ablated with 40% laser power, bottom left – 60%, upper right – 80% and bottom right- 100%.

Figure 5 represents fluorescence images of anti-chicken IgG AlexaFluor 546-conjugated deposited on the 'bar code' micro-structure fabricated in a Au-PMMA bilayer. From the left: 1st line, ablation with 100% of laser power at a rate of 20 pulse/s, and the writing speed of 10 μm/s; 2nd line, ablation with 100% of laser power at a rate of 20 pulse/s, and a writing speed of speed 10 μm/s, repeated twice; 3rd line, ablation with 100% of laser power at a rate of 20 pulse/s, and a writing speed of 20 μm/s. The inset represents a pseudo-map of the intensity of the fluorescence.

Figure 6 represents topographical (top left) and friction force (top right) images of a channel created by the laser beam (100% laser power, 20 pulse/s and 10 μm/sec writing speed). The bottom plot represents the profile of a transversal section of the channel.

Figure 7 represents fluorescence images of labelled protein adsorbed on structures fabricated via laser ablation at different power levels (conditions as in Figure 6). The amplification of fluorescence (in inset on each line) and the pseudo-map of the intensity of

the fluorescence (inset upper left) compared with the hydrophobicity map (inset upper right) reveal a 'fine structure' of protein deposition, preferentially on the hydrophilic edges of the channel and on the hydrophobic ridge on the center of the channel.

Figure 8 shows detection of specific antigens in high density 'bar code' array format demonstrated by incubation of the array with fluorescently labelled individual or collective antibodies. On the top – a fragment of 'bar code' array of two different proteins in the bright field; on the bottom – fluorescent image of the same array with specific recognition by anti chicken IgG AlexaFluor 546 conjugate.

Examples

Atomic Force Microscopy (AFM) can be used not only for fine mapping of the topography of a surface, but also for probing the physics and chemistry of the surface. In this context, AFM has been used to probe intermolecular interactions with pN sensitivity and spatial resolution of nanometers, Noy *et al.* (1997). When imaging under ambient conditions, the capillary condensation between the tip and sample surfaces reflects the relative degree of hydrophilicity and can be used as a basis for discriminating between hydrophobic and hydrophilic groups, Wilbur *et al.* (1995). The image contrast in a lateral force map is effectively a measure of tip-to-surface friction. Frictional force follows the generalized Amonton's law, Noy *et al.* (1997), Wilbur *et al.* (1995), Sinniah *et al.* (1996), Vezenov *et al.* (1997):

$$F_f = \mu F_N + F_o \qquad (1)$$

Where μ is friction coefficient, F_N is the lever-induced normal force and F_o is 'residual force' which correlates with adhesion force between the tip and the sample surfaces.

Previous studies, Noy *et al.* (1997), have shown that the interaction forces between tips and samples which both terminate with hydrophobic groups are small. Observed interaction forces are also small when one of the surfaces terminates with hydrophobic groups and the other terminates with polar groups, whereas significant interactions are observed when both the tip and sample surfaces terminate with hydrophilic groups (hydrogen-bonding).

The SiN_4 tip used in the present study is hydrophilic due to the native oxide surface layer. The frictional force is therefore higher as the tip is scanned across a hydrophilic surface, compared to a hydrophobic surface.

Protein Preparations: Several immunoglobulins (IgG's), i.e. bovine IgG, chicken IgG, human IgG, rabbit IgG, and affinity isolated antigen-specific corresponding antibodies (whole molecule) were purchased from Sigma. Streptavidin and fibrinogen were used as control proteins. The IgGs were prepared as stock solutions at a concentration of 2 mg/ml and diluted with TBS to 100 μg/ml as working solutions prior to experiments.

Three fluorescent labels have been used, namely: fluorescein isothioiocyanate (FITC), AlexaFluor 456, and AlexaFluor 350. The FITC and AlexaFluor fluorescent tags have been conjugated to the selected proteins using FluoroTag Kits purchased from Sigma and Molecular Probes, respectively. The labelling procedure was carried out according to the instructions of the manufacturer. Each protein was used in concentration of 2 mg/ml. The labelled proteins were purified from unconjugated fluorescent dyes using a Sephadex G-25 column. The concentration of antigen conjugate was determined by UV-Vis spectroscopy. The Fluorescent dye/Protein molar ratio of the purified protein was determined by measuring the absorbance at 280 nm (for protein), and 495 nm (for FITC), 556 nm (for AlexaFluor 546), and 346nm (for AlexaFluor 350).

Preparation of the micro-fabricated structures. Glass slides or cover slips (0.17 mm thick, 24 x 24 mm, Knittel) were sonicated in Nanopure water for 30 min and washed copiously with filtered (0.2 μm) Nanopure water (18.2 MΩ/cm), dried under a stream of high purity nitrogen, and then primed with hexamethyldisilazane. A 4 wt% solution of PMMA in propylene glycol methyl ether acetate (PGMEA) 99% (purchased from Sigma Aldrich Co.) was spin-coated at 3000 rpm for 40 s using a Specialty Coating Systems spin coater (Model P6708). For these conditions, the PMMA film thickness was 0.5 μm. The coated substrates were then soft baked at 85°C for 30 min, and stored in a desiccator prior and after gold deposition. The deposition of gold was done using a sputtering SEM-coating unit E5100 (Polaron Equipment Ltd) at 25 mA for 90 s at 0.1 Torr. For these conditions,

the gold film thickness was 50 nm. The gold-layered substrata were then incubated with bovine serum albumin (BSA) by immersion in a 1% w/v BSA 10 mM phosphate-buffered saline (PBS) solution (pH 7.4) at room temperature for approximately 1 h, and then rinsed with PBS followed by Nanopure water.

The laser-based microfabrication of gold-coated polymeric films can be readily accomplished with commercially available microscope adaptations. The system (Cell Robotics, Inc.) comprised a Nikon Eclipse TE300 inverted microscope, coupled with a computer-controlled, pulsed nitrogen laser emitting at 337 nm with a maximum intensity of 120 μJ/pulse and focused directly through the microscope objective lens.

Quantification of surface-related processes. The hydrophobicity of the films was estimated by contact angle measurements. Advancing contact angles were measured on sessile drops (2 μl) of Nanopure water at room temperature (20-23°C) in air using a contact angle meter constructed from an XY stage fitted with a (20 μl) micro syringe, a 20x magnification microscope (ISCO-OPTIC, Germany) and a fibre-optic illuminator. The observed values were averaged over six different readings.

Atomic Force Microscopy (AFM) was carried out on an Explorer system (ThermoMicroscopes) in the normal contact mode. The AFM system is based on detection of tip-to-surface forces through monitoring optical deflection of a laser beam incident on a force-sensing/imposing lever. Several scanners were used in order to cover the scales of lateral topographical and chemical differentiation; the fields-of-view ranged from 100x100 μm down to 8x8 μm. The analyses were carried out under air-ambient conditions (temperature of 23°C and 45% relative humidity). Pyramidal-tipped, silicon nitride cantilevers with a spring constant of 0.032 N/m were used. As the tip is scanned across the surface, the lateral force acting on the tip manifests itself through a torsional deformation of the lever, which is sensed by the difference signal on the Left-Right signal on the quadrant detector. The difference signal can be plotted as a function of XY location in the topographical field of view, and the resulted friction force image can then be correlated directly with the topographical image.

The attachment of fluorescently labelled proteins on the ablated micro-structures was visualized using and analysed using two different microscopic systems. The first is a Nikon TE300 inverted microscope, coupled with an epi-fluorescence illumination unit fitted with filter sets specific towards FITC (CR101, Chroma Technology) and AlexaFluor (XF108-2, Omega Optical, Inc.). The second was a Nikon Microphot FX microscope with a UV light source (Nikon Mercury Lamp, HBO-100 W/2; Nikon C.SHG1 super high pressure mercury lamp power supply) at 100X objective. These images were captured on a Nikon camera (FX-35WA). The fluorescent images were observed using an intensified CCD video camera, Lumi Imager (Photonic Science), and processed using PaintShop Pro (Jasc Software). The fluorescence intensities were analysed using Gel-Pro Analyser software, version 4.0.

Multi-analyte antibody assay. The assays fabricated as described above comprised different IgGs (1-7 μl of 100 μg/ml), either fluorescently labelled for the visualization of the selectivity of protein attachment, or unlabelled for the visualization of the selectivity of protein recognition by labelled antigens, deposited onto micropatterned ablated areas as described above.

For assay fabrication and process monitoring and optimization, IgG conjugates with FITC and AlexaFluor's (2-7 μl of 100 μg/ml) were deposited onto fabricated ablated geometries either in a 'blanket' mode, flooding the whole surface of the assay; or in a spatially-addressable manner, using the pico-liter pipette. For the 'blanket' deposition, the slide was incubated for 30 min at room temperature in a humid chamber, then the slide was washed three times with PBS and twice with Nanopure water. The spatially-addressable deposition used a pico-liter pipette (CellSelector module, Cell Robotics Inc.) mounted on the same precision XY stage. Very small amounts, usually around few hundreds nanoliters down to hundreds of picoliters, can be deposited in precise locations, usually within micron-range precision.

For the testing of the assays, the IgGs-covered surfaces were incubated individually or collectively with corresponding fluorescently labelled antibodies and control proteins (e.g. fibrinogen, streptavidin) for 2 h at room temperature. The assay structures were then washed three times with PBS, and twice with Nanopure water. The images of the selectively recognized patterned features were analysed as described above.

Example 1: Assays in array format

In order to explore the interaction between laser power – surface properties - molecule adsorption, and in particular, protein adsorption, 50 x 50 μm areas were ablated at different ablation energies. A few lines were also ablated to form channels. Fluorescently labelled antibody (anti-chiken IgG AlexaFluor 546) was deposited and incubated. The results are shown in Figure 3. It appears that the proteins deposit primarily on the regions at the edges of the ablated areas and that, after a certain power threshold (around 50%), this concentration levels off. In principle, the higher concentration of the protein could be an artefact resulting from the verticality of the wall (apparently thicker protein layer seen from the top of a vertical wall). However, the height of the wall is not large (around 50 nm) and, more importantly, the AFM analysis (Figure 4) points out the real differences in the material characteristics near the edges of the ablated area. First, the lateral force measurements, taken before protein deposition, proves that the outer surface (Au) has a similar hydrophobicity with the inner area (PMMA) in line with the similar contact angles for these two materials (around 65° and 70°, respectively). Second, the AFM-measured topography shows that indeed the bottom of the well is deeper and rather flat, except for the edges that are elevated above the level of the gold layer. Third, the AFM-mapping of the lateral force clearly shows a hydrophilic rim at the edges of the ablated area (areas in Figure 5, right side), possibly guarded by thinner hydrophobic stripe (brighter and darker areas in Figure 5 right, respectively). Moreover, the width of the rim seems to be rather independent of the laser power.

Example 2: Bar code assays.

Linear structures which both decrease the actual amount of protein used for deposition, especially if a spatially-addressable deposition is used, as well as increase the capacity for

miniaturization in a lateral if not in a 2D manner, were fabricated. Another benefit of this approach arises from the possibility to encode the information (e.g. type of antibody, concentration) through a combination of vertical lines in a 'bar code', 'informationally-addressable' mode and not in a 2D, spatially-addressable mode like in the classical arrays. The results also demonstrate, *inter alia*, the complexities of protein adsorption in fabricated channels, with the resolution of the variation of the protein concentration in the nanometer range. These complexities are likely to have an increasingly important impact in microfluidics, especially for devices that comprise nano-channels.

Proteins adsorb either via hydrophobic interactions between hydrophobic patches on the molecular surface and adsorbing surfaces, or via weaker electrostatic interactions between charged patches and charged surfaces. It follows that the protein will be adsorbed at the center of the well and on the porous zone at the edges. On rectangular ablated areas, where the center of the well is ablated by subsequent sweeps of the laser beam, much of the protein adhesion occurs at the edges of the ablated area.

The processing conditions (e.g. laser power, speed of writing) were tested in order to clarify the optimal surface treatment that will facilitate the best and reproducible protein attachment. The results of this experiment are presented on Figure 6. Protein attachment reached a maximum on the lines ablated with 100% of the laser power and at the highest pulse rate (i.e. 20 pulse/s, line no. 3 from the left in Figure 6). When using the same total energy, but via the ablation with a rate of 10 pulse/s repeated twice (lines no. 1 and 2 from the left, respectively) the protein adsorption was less apparent. Therefore further experiments used this optimized parameters for the fabrication of the protein patterns.

To understand the protein adsorption in ablated channels and compare with the adsorption on rectangular features, the inner surface of the channels were analysed using AFM (Figure 6). Apart from the hydrophilic elevated ridges observed before, the AFM analysis has revealed a hydrophobic elevated-from-the-bottom-of-the-channel line. The high resolution images of the fluorescence compared with high resolution AFM lateral force mapping (Figure 7) reveal a 'fine structure' of protein deposition, preferentially on

the hydrophilic edges of the channel and on the hydrophobic ridge on the center of the channel.

The high specific surface of the channel, which is caused by either the uneven bottom of the channel or by the possible porous material of the ridges, cooperate with the many variations of the surface hydrophobicity to allow a high concentration of diverse proteins in the ablated channels compared with rectangular ablated areas.

Example 3: Specific antibodies recognition.

The protein detection system described above was demonstrated by the incubation of the 'bar code' assay (fabricated as described above) with both IgG's and control proteins. For an example, Figure 8 presents a part of an array format with a 'bar code' structure that was functionalized with two proteins, before (top image, bright field) and after protein recognition (bottom image, fluorescence). Chicken IgG was deposited on a fragment of the 'bar code' structure (three lines on the right, Figure 7 top) and streptavidin was deposited on the rest of the structure (two lines on the left, Figure 7 top) using the picoliter pipette. The anti-chicken IgG deposited over the whole array of specifically recognized the chicken IgG lines (bottom image).

References

Andrade, J. D.; Hlady, V. *J. Biomate. Sci. & Polymers* **1991,** *2,* 161-72.

Bernard, A.; Delamarche, E.; Schmid, H.; Michel, B.; Bosshard, H. R.; Biebuyck, H.; *Langmuir* **1998,** *14,* 2225-2229.

De Wildt, R. M.; Mundy, C. R.; Gorick, B. D.; Tomlinson, I. M. *Nature Biotech.* **2000,** *18,* 989-994.

Fodor, S. P. A.; Read, J. L.; Pirrung, M. C.; Stryer, L.; Lu, A. T.; Solas, D. *Science* **1991,** *251*, 767-773.

Ismagilov, R. F.; Ng, J. M.; Kenis, P. J.; Whitesides, G. M. *Anal. Chem.* **2001,** *73,* 5207-5213.

McDonald J. C.; Metallo S. J.; Whitesides G. M. *Anal. Chem.* **2001,** *73,* 5645-5650.

Nicolau, D. V.; Taguchi, T.; Taniguchi, H.; Yoshikawa, S. *Langmuir* **1998,** *14,* 1927-1936.

Nicolau, D. V.; Taguchi, T.; Taniguchi, H.; Yoshikawa, S.; *Langmuir* **1999,** *15,* 3845-3851.

Nicolau, D. V.; Cross, R. *Biosens. & Bioelectron.* **2000,** *15,* 85-91.

Noy, A.; Vezenov, D. V.; Lieber, C. M. *Annu. Rev. Mater. Sci.* **1997,** *27,* 381–421.

Schwarz, A.: Rossier, J. S.; Roulet, E.; Mermod, N.; Roberts, V. A.; Girault, R. H. *Langmuir* **1998,** *14,* 5526–5531.

Sinniah, S. K., Steel, A. B., Miller, C. J., Reutt-Robey, J. E. *J. Am. Chem. Soc.* **1996,** *118,* 8925-8931.

Sundberg, S. A. *Curr. Opin. Biotech.* **2000,** *11,* 47-53.

Vezenov, D. V.; Noy, A.; Rozsnyai, L. F.; Lieber, C. M. *J. Am. Chem. Soc.* **1997,** *119,* 2006-2015.

Wadu-Mesthrige, K.; Xu, S.; Amro, N. A.; Liu, G. *Langmuir* **1999,** *15,* 8580 –8583.

Walter, G.; Bussow, K.; Cahill, D.; Lueking, A.; Lehrach, H. *Curr. Opin. Microbiol.* **2000**, *3*, 298-302.

Wang, C.; Oleschuk, R.; Ouchen, F.; Li, J.; Thibault, P.; Harrison, D.J. *Rapid Com. Mass Spectr.* **2000**, *14*, 1377-1383.

Wilbur, J. L.; Biebuyck, H. A.; MacDonald, J. C.; Whitesides, G. M. *Langmuir* **1995**, *11*, 825-831.

Zhao, X. M.; Xia, Y. N.; Whitesides, G. M. *J. Mater. Chem.* **1997**, *7*, 1067-1074.

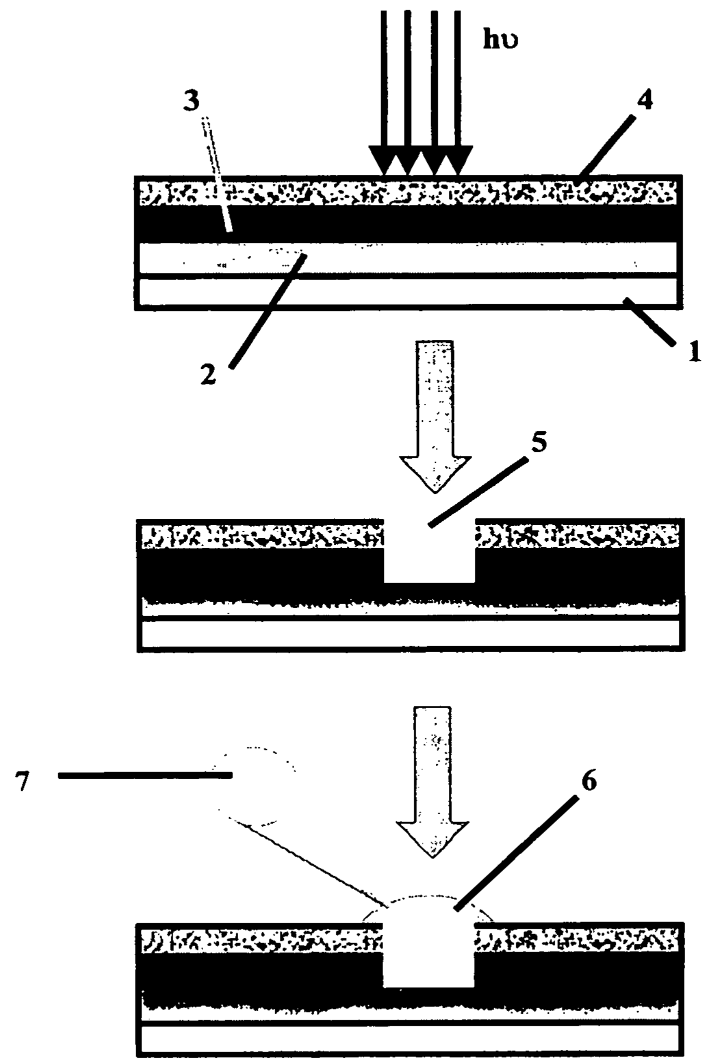


Figure 1.

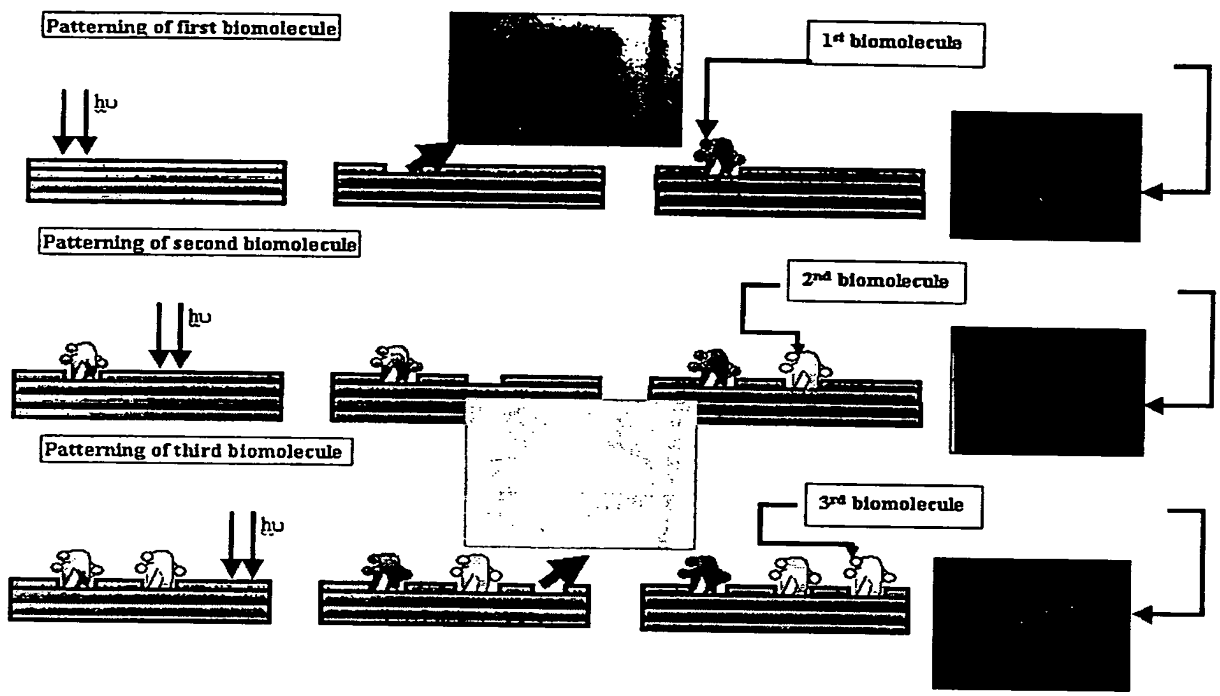

Patterning of first biomolecule
hυ
1st biomolecule
Patterning of second biomolecule
hυ
2nd biomolecule
Patterning of third biomolecule
hυ
3rd biomolecule

Figure 2.

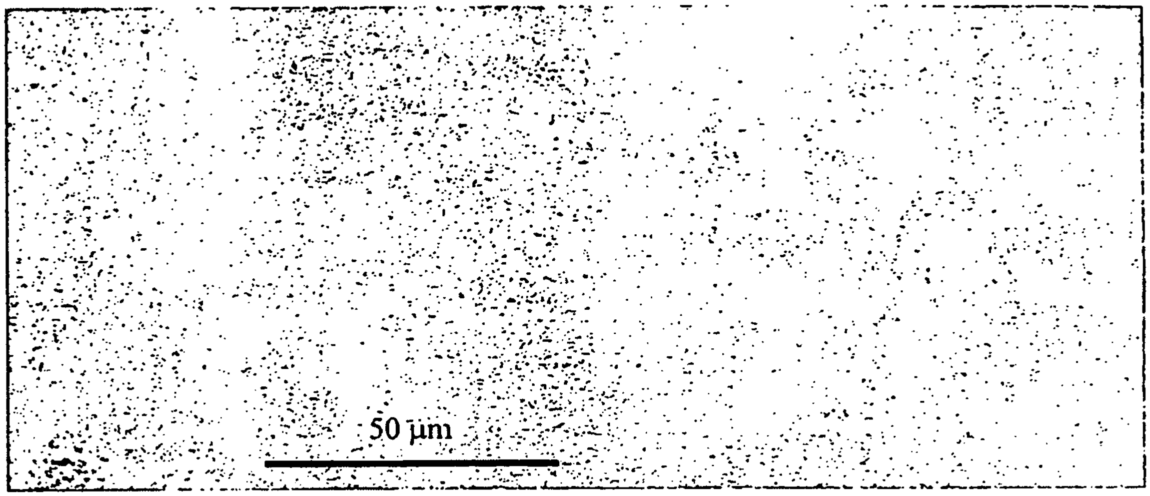


Figure 3

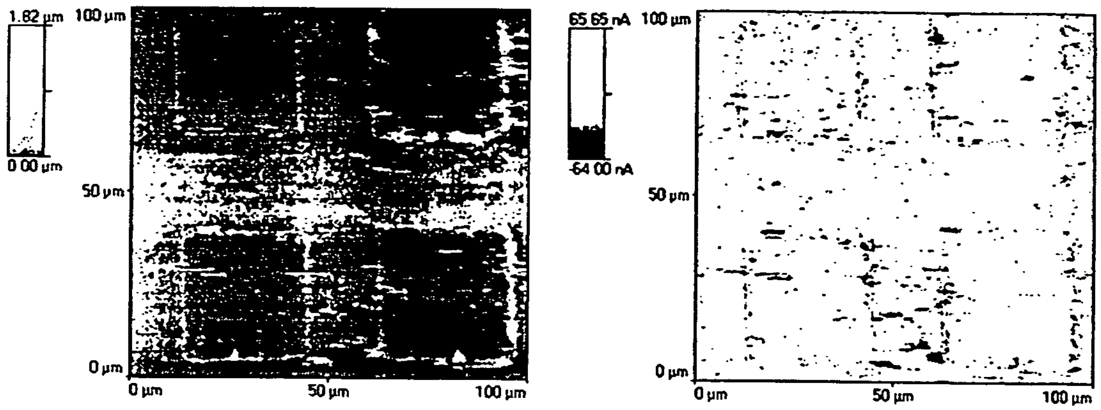

1.82 µm
0.00 µm
100 µm
50 µm
0 µm
0 µm
50 µm
100 µm
65.65 nA
-64.00 nA
100 µm
50 µm
0 µm
0 µm
50 µm
100 µm

Figure 4.

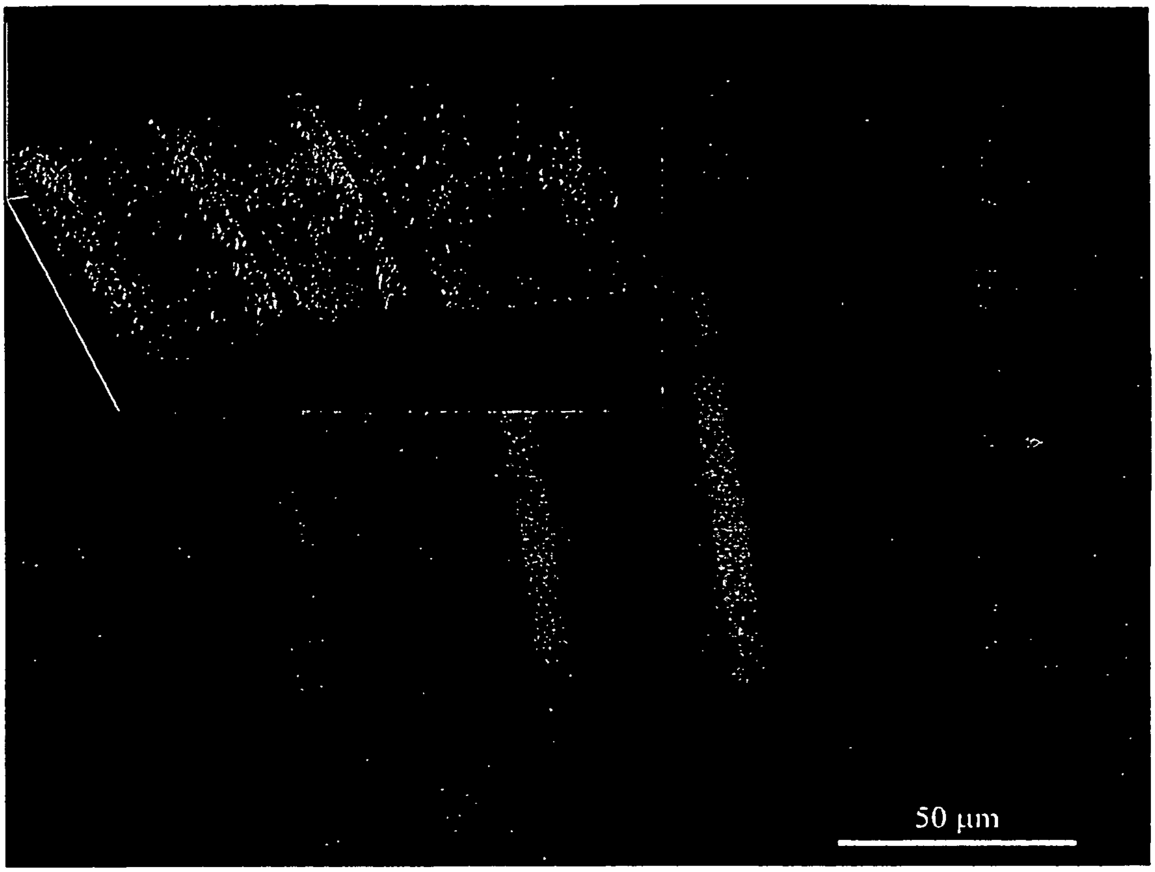


Figure 5.

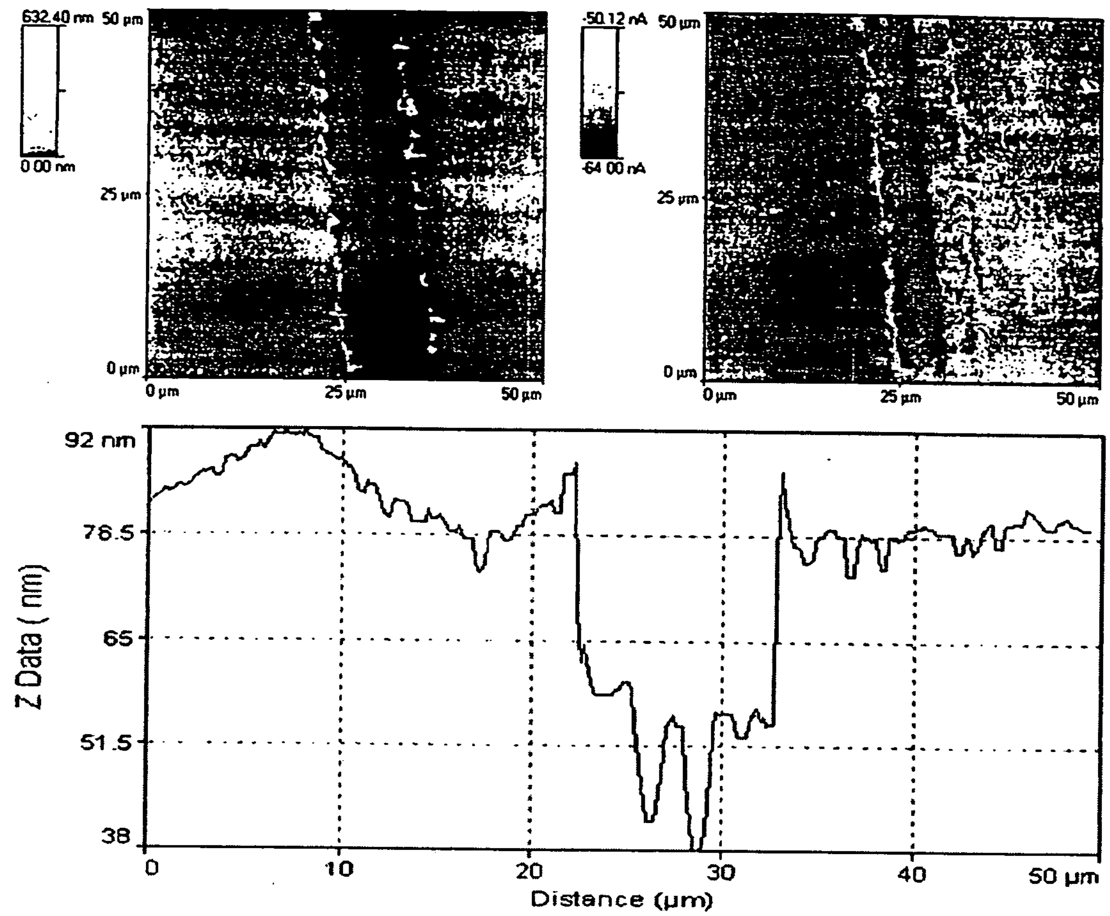
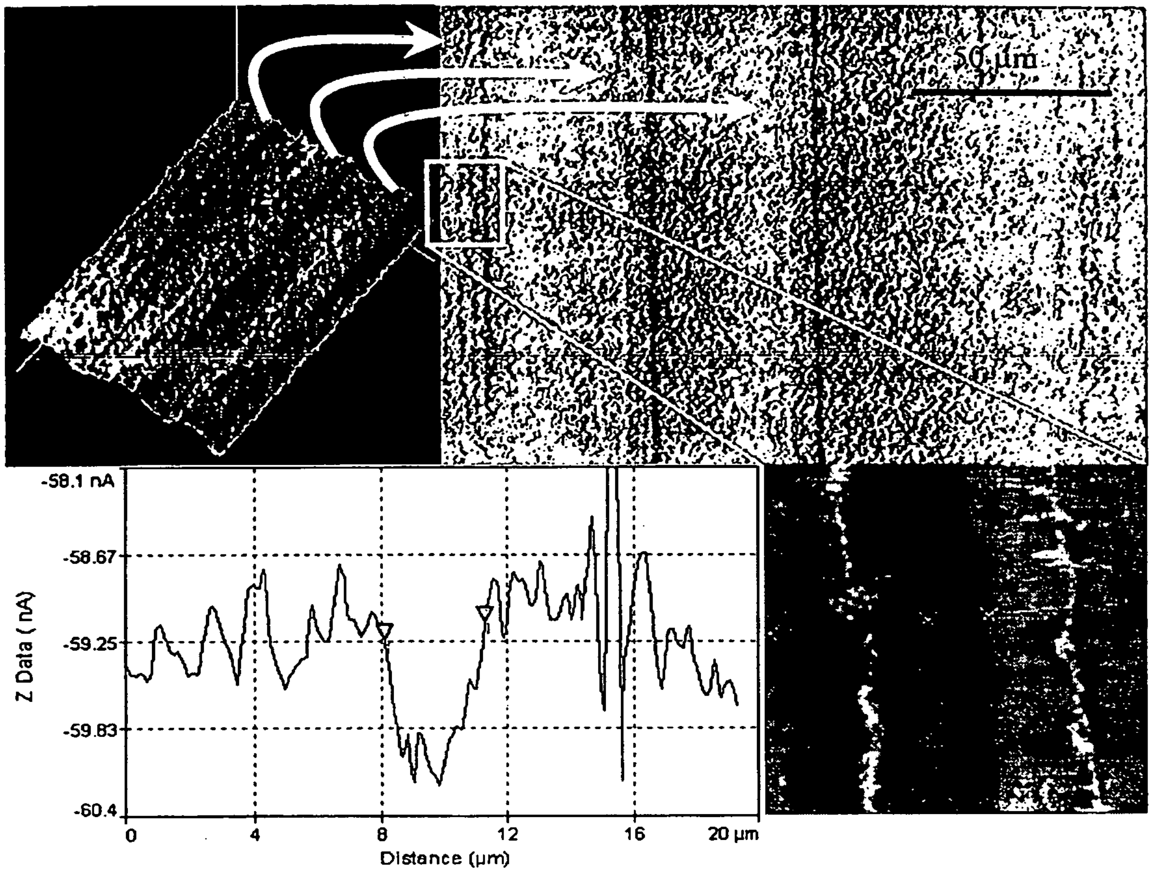

632.40 nm
0.00 nm
50 µm
25 µm
0 µm
0 µm
25 µm
50 µm
-50.12 nA
-64.00 nA
50 µm
25 µm
0 µm
0 µm
25 µm
50 µm
92 nm
78.5
65
51.5
38
Z Data (nm)
0
10
20
30
40
50 µm
Distance (µm)

Figure 6.



50 µm
-58.1 nA
-58.67
-59.25
-59.83
-60.4
Z Data (nA)
0
4
8
12
16
20 µm
Distance (µm)

Figure 7.

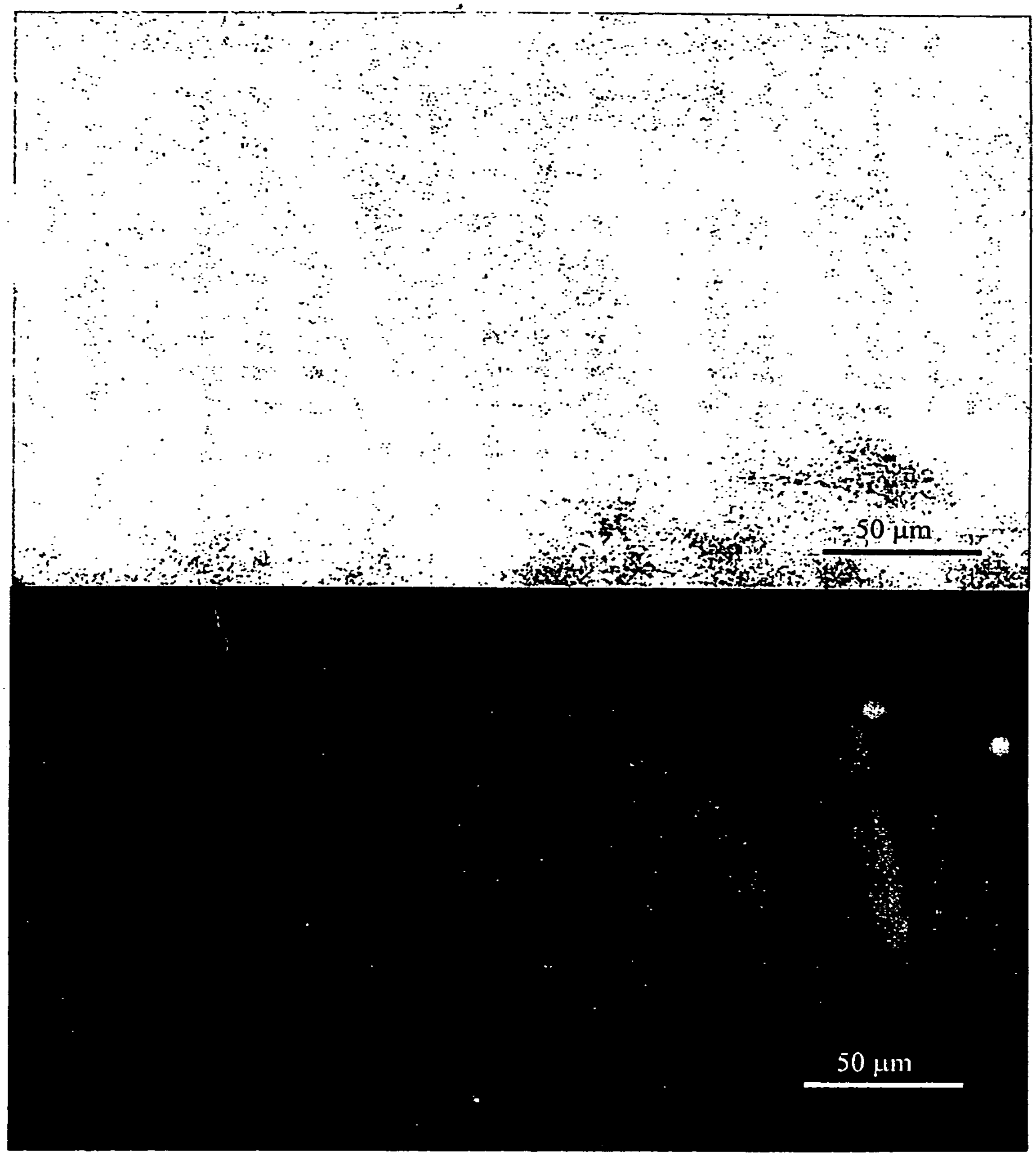


Figure 8